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CONTENTS

Progress in the Standardization of Stains

Dyes for Bacteriostatic Work H. J. CONN 1

Stains Recently Certified 2

The History of Staining

Anilin Dyes in Histology. H. J. CONN 3

Hematein—Its Advantages for General Laboratory Usage. S. I. KORNHAUSER 13

The Mechanism of Staining Explained on a Chemical Basis. II. General Presentation. ALLEN E. STEARN and ESTHER WAGNER STEARN 17

The Negative Staining of Bacteria. W. Ch. DORNER 25

Notes on Technic

Detecting Oil in Twigs. RACHEL HAYNES 29

A Wire Holder for Staining Slides. JOHN E. SASS 29

Meyer's Hemalum—A Correction. JOHN E. SASS 30

Laboratory Hints from the Literature 31

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Editor and Business Manager
H. J. CONN
Soil Bacteriologist, N. Y. Agr. Exp. Station

Consulting Editors
CHEMISTRY: W. C. HOLMES Color Lab., U. S. Dept. of Agriculture
ZOOLOGY: S. I. KORNHAUSER School of Medicine, Univ. of Louisville
PATHOLOGY: F. B. MALLORY Boston City Hospital
BOTANY: W. R. TAYLOR Univ. of Pennsylvania

SUGGESTIONS TO AUTHORS SUBMITTING MANUSCRIPT

Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

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Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

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STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

DYES FOR BACTERIOSTATIC WORK

The utilization of dyes for their bacteriostatic effects was a minor matter when the standardization of stains was begun. It has, however, been growing in importance each year with the possibility that it may soon be as important a use of dyes as their employment in staining. Brilliant green has been used in the study of the colon-typhoid group of bacteria for a good many years. Perhaps equally well known is the utilization of crystal or methyl violet for the separation of Gram-positive from Gram-negative bacteria and for cultivation of the tubercle organism in pure culture. Many of the other ways, however, in which this valuable property of dyes is now being utilized are less widely known—as for isolating the legume nodule organism from soil and for differentiating the various members of the contagious abortion group.

The increased employment of dyes in such work is a splendid advance, but a word of caution is necessary. The Biological Stain Commission does not yet test dyes for bacteriostatic work before issuing certification on any batch, except in the case of brilliant green, whose use in the isolation of the typhoid organism is definitely specified on the label. Certain dyes with particularly interesting bacteriostatic properties have been frequently tested in the laboratory of a Commission member; but recent studies indicate that the subject is so complicated that much more work must be done before positive statements can be made as to bacteriostatic titres. Certified stains have ordinarily been tested by the Commission *as stains only*, and no guarantee is made that any two batches of the same dye will have the same inhibiting effect on bacteria. Anyone, therefore, who publishes work in which dyes have been used for bacteriostatic purposes should base any statements upon comparative results with several batches of any one dye, and should give the name of the manufacturer of each, the dye content if stated on the label, the certification number if there is one, as well as any other data helping to identify the sample employed. This is necessary to enable other investigators to check up on the findings.

There are two reasons why dyes are not yet standardized for bacteriostatic purposes, as well as for staining. In the first place, testing any particular batch for this purpose would require so much work as to be a research problem in itself, and the Commission does not have sufficient assistance for such an undertaking. In the second place, there seems to be an impression in some quarters that a dye satisfactory for bacteriostatic use may be employed as a therapeutic agent. This, of course, involves many other questions, including toxicity to human beings, upon which the Stain Commission, as a non-medical body, has no authority to pass. There is some danger that approval of a dye for bacteriostatic use might be interpreted as an endorsement for therapeutic purposes. To avoid this misunderstanding, the Stain Commission has kept out of this field. It is realized, however, that more standardization along this line should be undertaken and it is hoped that means to do so can be found in the future.

H. J. Conn

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the October number of this journal.

Stains Certified August 15 to November 15, 1929*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Erythrosin	NEr 1	92%	As histological stain	Aug. 26, 1929
Brilliant cresyl blue	NV 7	67%	As special blood stain	Sept. 7, 1929
Fuchsin, acid	NR 6	70%	As histological stain and for use in Andrade indicator	Sept. 18, 1929
Fuchsin, basic	NF 16	82%	For staining and for use in the Endo medium	Oct. 2, 1929
Eosin Y	NE 6	83%	As histological stain	Oct. 17, 1929
Eosin Y	LE 5	74%	As histological stain	Oct. 17, 1929
Hematein	FHi 1	——	As histological stain	Nov. 6, 1929

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

THE HISTORY OF STAINING

Anilin Dyes in Histology

H. J. Conn

About the middle of the last century, William Perkin, while still a mere boy, made his epoch-making discovery of an anilin dye, the result of which was a revolution not only in the dyeing industry, but in microscopic science as well. The story of how he tried unsuccessfully to synthesize quinine, but attracted by the violet stains which he noticed, was sidetracked from his investigation and isolated the first anilin dye, has been told so many times that it need not be repeated here. He called this dye "mauve" and it was the first synthetic dye to be manufactured on a commercial scale. Strange to say, the exact date of Perkin's discovery does not seem to be known. The only publication of this discovery was apparently the patent which he very promptly took out. The date of the patent was 1856, but as some authorities give the date of his discovery as 1854, there is some possibility that it may have preceded the patent by two years. In 1854, however, he was only sixteen years old and it is difficult to credit such a young boy with such an important discovery. After Perkin had shown the way, the discovery of other dyes followed rapidly. Basic fuchsin was first manufactured the same year as Perkin's patent (1856). The discovery of various other dyes which now have biological use occurred as follows: safranin 1859, methyl violet 1861, anilin blue W. S. 1862, eosin and methyl green 1871, thionin and methylene blue 1876, acid fuchsin 1877, orange G 1878, Sudan III 1880, crystal violet 1883.

Perkin would undoubtedly have been much surprised if anyone had hinted to him at the time what an influence the discovery of anilin dyes was going to have on microscopy. It was not long, however, before microscopists saw the possible significance of these dyes and began employing them. The first suggestion of their use in histology seems to have been made by Beneke (1862) who employed acetic acid colored with a dye which he called "lilac anilin." The dye which he thus designated was a commercial product undoubtedly impure and very difficult to identify in the terms of the dyes as we know them today. Probably, however, it was the same dye as that discovered by Perkin, which was known in those days under various commercial names such as anilin violet, anilin purple, purpurin or tyrian purple.

The following year, however, Waldeyer (1863) employed a greater variety of anilin dyes. He mentions three which he calls: rosanilin (anilin red), Paris blue (anilin blue) and anilein (Wilm.) (anilin violet). His Paris blue was undoubtedly an acid dye, his anilein quite possibly the same as the lilac anilin employed by Beneke. His rosanilin was beyond question one of the basic fuchsins, altho not necessarily pure rosanilin. He observed particularly the ability of this red dye to stain nuclei more deeply than cytoplasm and the axis cylinders more so than the medulary sheaths of nerves.

Two years later Onimus (1865) definitely named fuchsin in describing the dye which he employed in histological technic and gave its chemical formula as $C_{20}H_{19}N_3H_2$, which is very close to that of rosanilin. On the other hand, he says that the dye is decolorized by bases, either in solution or in the tissue after staining. He states that one may dissolve fuchsin in a potash solution and obtain a colorless liquid; pieces of tissue placed in this may be given color under the microscope by adding a drop of acid. This statement is hard to reconcile with the behavior of basic fuchsin, and suggests that he is describing a property of acid fuchsin. On the other hand, he states that the dye stains nuclei in particular and also other granules in the cell. This statement suggests a basic dye; furthermore acid fuchsin is not supposed to have been known until 1877. All this shows how difficult it is to tell when some of these dyes were actually first employed in microscope work.

It is also interesting to note that Onimus injected this dye into living pigeons and rabbits. He found that the animals survived the treatment, but did not obtain any noteworthy staining results. This is beyond question the first use of an anilin dye as a vital stain.

Frey (1868) about this same time employed a dye which he called "parme." He gives its method of preparation as the treatment of diphenyl rosanilin with sulfuric acid. This shows beyond any question that it was an acid dye; one of the water soluble anilin blues. This may or may not have been the same anilin blue obtained by Waldeyer five years earlier, altho the probabilities are that the latter was actually using something different, but in the same group of dyes.

Böttcher (1869) seems to have employed a basic dye at about the same time, rosanilin nitrate, i.e. one of the fuchsins. Of even greater significance in Böttcher's work is the fact that he introduced the principle of differentiating with alcohol, either following rosanilin or carmin.

Some anilin blue and some dye called dahlia were employed a little later by Zuppinger (1874), anilin red by von Ebener,[1] Merkel (1870) and Jackson (1874). The latter referred to the dye as magenta, and by discussing its chemistry shows it to have been basic fuchsin.

Huguenin (1874) employed a dye which he called "dahlia" for staining axis cylinders of nerves. Dahlia being an indefinitely known dye today, naturally one can merely be certain that this author was employing some violet dye.

Fischer (1876) apparently introduced the use of eosin for staining various kinds of tissue. He definitely designated the dye as the potassium salt of tetrabromfluorescein. He stained sections ten to twelve hours with an aqueous solution of this dye. He preferred, however, staining with an alcoholic solution of the color acid which he obtained by precipitation with acid from the aqueous solution. He described the picture obtained with this dye in some detail.

Not everyone at this period was equally enthusiastic about the anilin dyes. Tait (1875) as we have already seen (Stain Technology 4, 40) condemned all anilin colors and most solutions of carmin as coloring the whole mass too indiscriminately. This would suggest that he had employed only the acid dyes which as we have seen were the type in most general use at this period. No one, in fact, appreciated at that time what a radical distinction there actually is between the nature and behavior of acid and basic dyes.

Various papers appeared from 1875 on, describing the use of anilin dyes in histology. It is hardly necessary to refer to them all, but some of those which seem most significant should be mentioned. A very interesting contribution which appeared this same year was that of Cornil (1875) on a dye which he called methyl anilin violet. There seems little question but that he was employing a triphenylmethane dye, and from his findings one would conclude that it was a basic dye. If this is the case, he probably was using one of the dyes of the methyl violet group. How similar this may have been to the dahlia employed by Huguenin the preceding year there seems to be no way of determining. The interesting point in Cornil's paper is that he seems to have been the first to observe metachromatic effects, at least with anilin dyes. He observed that hyaline cartilage was stained in such a manner that the cells and their capsules were violet, the intracellular material red; connective tissue fibrils and elastin fibers as well as the cells and fibers of elastic cartilage stained violet. He was quite enthusiastic over the differentiation thus obtained, but his interpretation of the matter is different from any modern theory of

[1]In 1870. Original not seen, cited from Gierke (1884).

metachromasy; it is well summed up in the title of his paper, the translation of which is: "On the dissociation of methyl anilin violet and its separation into two colors, under the influence of certain tissues, both normal and pathological, and particularly by the action of the tissues undergoing amyloid degeneration."

This same year saw the appearance of quite an important paper by Hermann (1875) who is often given the credit of introducing the principle of differentiation with alcohol. He is incorrectly credited with this as shown by Mann (1902), for Böttcher had already introduced this principle in 1869. Böttcher and Hermann both employed alcoholic differentiation after staining with a basic fuchsin. Hermann undoubtedly managed to present the work in such a way as to bring the method more distinctly to the attention of other histologists than had Böttcher. Undoubtedly, however, the real reason for the credit given to Hermann is that the method was taken up six years later by the famous histologist and cytologist, Flemming (1881), who called the procedure in his title "The Hermann nuclear staining procedure." The real importance of the procedure probably was not appreciated until it was developed by Flemming.

Hermann apparently did not use the principle of differentiation in connection with double staining effects; but the idea of double staining was introduced at about the same time as this paper of his. Double staining was not a new idea at this period as Schwartz (1867) had introduced the principle by staining with ammonium carminate followed by picric acid, while Ranvier (1868) combined these two dyes into one solution, which was subsequently much employed under the name of picrocarmin. Strelzoff (1873) employed carmin in combination with hematoxylin. Gerlach (1872) had used picric acid following alum hematoxylin. It was, however, the period between 1875 and 1880 that saw the introduction of the more complex anilin dyes such as eosin and the triphenylmethanes into double staining combinations. Poole in 1875 employed hematoxylin with anilin blue. Duval (1876) used carmin with anilin blue, while the following year Lavdovsky (1877) employed picric acid with eosin in a single solution. Calberla (1877) seems to have been the first to try an acid and a basic anilin dye together. For this purpose he used a mixture of eosin and methyl green dissolved in 30% alcohol. Sections stained in this fluid showed lymph cells blue to blue green, striped muscle fibres red, their nuclei green, smooth muscle fibres green, and their intracellular substance red. The following year Schiefferdecker (1878) used eosin in combination with various other anilin dyes such as dahlia, methyl violet and some anilin green other than methyl

green. He did not use the two dyes in the same solution but stained first with eosin and then with the other dye.

Three other outstanding papers during the latter half of the seventies were those of Ehrlich (1877) and (1879a and b). In the first of these papers he made the first systematic study of various anilin dyes for staining purposes that appears in the literature. It is a little easier to identify these dyes than in the case of those employed by earlier microscopists. Among these dyes it is interesting to notice that Ehrlich mentions safranin. Altho this dye had been introduced into the industry in 1859 no biologist seems to have employed it before Ehrlich. Many of Ehrlich's dyes are difficult to identify, as for example those which he called "primula" and "dahlia," the latter being the dye with which he did most of his work. Today primula and dahlia are terms used in the dye industry for mixtures of basic fuchsin and methyl violet, in other words water-soluble dyes, rosanilins or pararosanilins of varying degrees of methylation. Ehrlich, on the other hand, describes dahlia as monophenyl rosanilin, in distinction from parme blue which he calls diphenyl rosanilin and anilin blue which he claims to be triphenyl rosanilin, compounds which actually occur in varying proportions in the different shades of the alcohol soluble dye which we now call spirit blue. Ehrlich was evidently slightly mistaken in the matter, for these compounds are seldom if ever found free from one another. Possibly he was entirely mistaken in calling his dahlia a phenyl rosanilin; probably, however, that statement was correct and what Ehrlich actually used was one of the reddish shades of spirit blue rather than what we call dahlia today. This is further borne out by Ehrlich's comment that the usual form of dahlia on the market was alcohol soluble, altho a water soluble form had also been secured; this latter was probably an ethylated or methylated rosanilin such as we think of under the term dahlia today. The other dyes named by him were iodine violet, methyl violet, fuchsin, and a "red anilin dye purchased under the name of purpurin." In this latter he was very likely using Perkin's dye "mauve," sold under a different name.

Ehrlich employed these dyes in a 33% alcohol containing 7.5 or 12.5 cc. of glacial acetic acid per 150 cc. of the solution. Employed in this way he noticed that safranin and fuchsin stain quite differently from the other five dyes. These dyes stain not only the plasma cells but also the surrounding tissue, altho not quite so intensely. The other five dyes, on the other hand, stain only the cells. This distinction apparently was not between acid and basic dyes, as some of these five latter stains were apparently basic and others acid.

In his later paper (1879a), however, he fully recognized the distinction between acid and basic dyes. He seems to have been the first to realize that the dyes fall into these two groups, which are distinct both chemically and histologically. He showed very clearly the different behavior of these two groups of dyes when employed for staining blood, recognizing the distinction between the acidophile and basophile leucocytes. At the same time he observed that the granules in certain other leucocytes did not stain with either basic or acid dyes, and he wondered if they might not be colored by dyes of a different class. He followed up that idea and published the results in a slightly later paper (1879b) in which he proposes the use of "neutrale Farbkörper," a term which is best translated "neutral stains" because dye chemists employ the expression *neutral dye* in an entirely different sense. The neutral stains mentioned by Ehrlich in this paper are rosanilin picrate, and the acid fuchsin salts of methylene blue and methyl green respectively. He ran onto the difficulty that the neutral stains are practically insoluble in water, but discovered that they could be brought into solution in an excess of the acid dye. Accordingly he secured either of the acid fuchsin compounds above mentioned in aqueous solution by furnishing an excess of acid fuchsin. With such a solution he demonstrated the existence of neutrophiles.

The importance of this series of papers by Ehrlich can hardly be exaggerated. Beside his extremely important contribution to the knowledge of blood cells, he established the difference between acid and basic dyes for staining purposes, recognized the neutral stains, showed one method of bringing the latter into solution, and employed one of them as a blood stain over a decade before Romanovsky used a mixture of eosin and methylene blue for the same purpose. It is interesting, however, to notice that Gierke's splendid review of the subject (1884–5) was too nearly contemporaneous with Ehrlich's work to allow a full appreciation of the latter; for altho these accomplishments of Ehrlich's are all listed, so little is said about them as to suggest that Gierke did not suspect what important contributions they actually were.

The outstanding developments of this field during the eighties are rather less numerous than those of the seventies; most important was the work on the azure group of dyes. Bernthsen (1885) made his well-known study of the oxidation products of methylene blue and called one of them methylene violet; while Ehrlich (1886) carried on important histological work with these dyes and with the closely related thionin. Altho Bernthsen's conclusions as to the chemistry

of this group have to be accepted today with some reservations (see MacNeal, 1925), his paper was an important advance at the time. According to Mann (1902) Ehrlich first used thionin in 1885, in which year he was also the first to employ orange G; reference to this work has not yet been located, however. In 1886 Griesbach was apparently the first to use congo red for histological work.

One important paper was published just at the very end of this decade, altho the technic in question was really developed in the nineties rather than the eighties. VanGieson (1889)[2] employed the following mixture:

Sat. aqu. sol. acid fuchsin Grübler.....few drops
Sat. aqu. sol. picric acid.....100 cc.

after deep staining of Müller fixed material in hematoxylin (e.g. Delafield's) as a differential stain for nervous tissue. Ernst (1892) employed this same technic (citing VanGieson's paper as its source) for staining connective tissue. The VanGieson stain seems to have been the first triple staining procedure in the literature.

At about this same time Unna (1891) proposed polychrome methylene blue. He recommended three formulae for methylene blue; of these the one which is generally known today as Unna's formula is:

Methylene blue.....1.0
K_2CO_3(Na_2CO_3 or $(NH_4)_2CO_3$).....1.0
Distilled water.....100.0

This (used in high dilution) he recommended for staining mast cells red with plasma cells blue. For the differentiation of all cells and intercellular substances he recommended the same formula with the addition of 20 parts of alcohol; while for staining plasma cells he substituted 0.05 parts of KOH for the carbonate. He says nothing about the need of ripening the solution; altho modern textbooks always specify that the solution must stand some time to insure proper polychroming.

Another important procedure published this same year was the Flemming (1891) triple stain, calling for successive staining with safranin, "gentiana" and "orange." This is used principally in cytological work and will be discussed in the next paper. Benda (1891) the same year proposed his well-known procedure with safranin followed by light green SF yellowish, another technic used primarily in cytological work.

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[2]This reference is generally given incorrectly. It has been finally located only after considerable search thru the literature.

The Pappenheim (1899) methyl-green-pyronin stain was first published at a little later period. At this time it was recommended as a stain for lymphocytes in the following proportions:

Methyl green	3–4 parts
Pyronin	1–4 parts

Application of this stain was followed by staining with "scharlach."

Mallory (1900) proposed the well-known anilin blue connective tissue stain. Corrosive sublimate or Zenker material in paraffin or celloidin was stained in 0.05-0.1% aqueous acid fuchsin, then treated in 1% aqueous phosphomolybdic acid and stained in the following solution:

Anilin blue	0.5 g.
Orange G	2.0 g.
Oxalic acid	2.0 g.
Water	100.

Among the other procedures originating at about this time were those employing the fat stains. Sudan III was used by Daddi (1896) for staining fat globules in sections. Michaelis (1901) seems to have been the first to obtain a good conception of the principles of fat staining and discussed the chemistry of various oil soluble dyes. On the assumption that a dye of the same structure as sudan III, but with methyl groups introduced into the molecule should behave similarly but give deeper staining, he tried the dye called scarlet R. This dye, under this name or that of sudan IV, is well known today, probably as the most satisfactory fat stain. In staining with it, Michaelis treated sections of formalin material 15 to 30 minutes in a saturated solution of the dye in 60 to 70% alcohol. The same year Herxheimer (1901), familiar with Michaelis' work, proposed a slightly different solution of sudan IV for the same purpose:

Sat. sol. of dye in abs. alc	70 parts
Water	10 "
10% aqueous NaOH	20 "

Following this time, of course, there have been many important staining procedures introduced; but with the beginning of the century the general principles had been established. Later procedures were ordinarily only modifications of those already in use.

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*Thus spelled in the author's title.

HEMATEIN—ITS ADVANTAGES FOR GENERAL LABORATORY USAGE

S. I. KORNHAUSER, *University of Louisville Medical School, Louisville, Ky.*

ABSTRACT—**The advantages of hematein over hematoxylin are that it is easy to prepare, easy to use, and saves time; while it gives equally good results. The writer has been employing for some time a pre-war imported hematein and until within the last few months has been unable to locate a satisfactory product of recent manufacture, either domestic or foreign. At the request of the Biological Stain Commission, however, an American manufacturer has at last put on the market a C. P. hematein which gives splendid results. The technic is as follows:**

Paraffin or celloidin sections of Bouin or Zenker-formol material are run down to water and stained about 5 minutes in Mayer's hemalum (0.5 g. hematein ground up in a glass mortar with 10 cc. 95% alcohol and added to 500 cc. of 5% aqu. sol. potassium alum.) Rinse 1 to 3 seconds in tap water. Dip 1 to 3 seconds in eosin B (1 part 0.5% sol. in 20% alc. added to 2 parts dist. water; filtered from time to time). Wash several minutes in running water or in several changes of tap water. Dehydrate and mount; with unattached celloidin sections this may be done by running up to 95% alcohol, spreading on slide, blotting, wetting with absolute alcohol, draining and mounting in euparal.

Many hematoxylin stains have decided disadvantages for routine laboratory work. The formulae are often complicated and the solutions must age or be artificially ripened before they will stain. Many such solutions are not selective enough to be used as progressive stains, but the slides or sections must be overstained and then decolorized to bring out nuclear or other basophilic structures. In decolorizing, much detail is often lost.

The writer has been very partisan to Mayer's hemalum for years, hemalum made from hematein and not artificially ripened. The advantages are:

1. The stain is easily prepared. One-half gram of hematein is ground up in a glass mortar with 10 cc. of 95% alcohol and this is added to 500 cc. of 5% aqueous potassium-alum solution.

2. It is immediately ready for use, and requires no ageing or artificial ripening. Sodium iodate or other ripeners shorten the life of the solutions which, after a few months, turn brown and fail to stain.

3. The stain is selective and may be used as a progressive stain on both paraffin and celloidin sections. This does away with decolorization which often decreases the value of the slide by wiping out the faintly stained details of the cytoplasm or intercellular material. Mitoses stand out with wonderful clearness, as do centrosomes, basal granules, axial filaments, etc.

4. It may be used to stain tissues in bulk for whole mounts or for sectioning.

5. It is as permanent as any hematoxylin stain.

To sum up, it is easy to prepare, easy to use, saves time, and gives good results.

For years the author has depended on some pre-war imported hematein, which was good. As this was used up, he tried some American hematein labelled "Hematin C. P.," which had not been submitted to the Commission for the Standardization of Biological Stains for certification. This proved very poor. Artificial ripening proved valueless. It gave a faint brownish stain to the whole section without picking out the nuclear material. Next "Hämatéin puriss" was imported. This was some better than the American product, but was very weak, altho selective. The author then tried to make Mayer's hemalum from ammonia hematein and from ripened hematoxylin in 95% alcohol. These proved less active than the pre-war hematein solution and were decidedly inferior.

With these discouraging results, the chairman of the Commission on the Standardization of Biological Stains was requested to get a good American hematein. The firm of MacAndrews and Forbes undertook this work. Beginning with refined hematoxylin, this was oxydized by the method of Engels-Perkin-Robinson and a net yield of 30% was obtained in three crops of 22, 6, and 2% respectively. Each of these crops was submitted to the author for trial. Mayer's hemalum was made up separately from each and tested within 12 hours after making the solution. Pre-war imported hematein was used as the standard of comparison. All sections used in all this testing were from the same block, a mouse testis and epididymis fixed in Bouin's fluid, embedded in paraffin and sectioned 10 μ thick. To be judged satisfactory a fresh solution had to give a strong selective nuclear stain in five minutes, and when counterstained with eosin bluish, according to our regular laboratory procedure, had to show details of structure, such as axial filaments of spermatozoa, chromosomes, centrosomes and basal granules of the epithelium of the ductus epididymis.

Lots 1, 2 and 3 all proved satisfactory and, in fact, lot 3 seemed the most brilliant and selective of all. Next, mixtures were made of 1 and 2, and then of all three and these were also satisfactory. All slides have been exposed, on the laboratory table to direct sunlight, when such was available, since September and the solutions have been kept in white glass bottles. So far sections and solutions are holding up well. It will still be many months until we know fully the keeping qualities, since we have, as yet, no test which will, in a short period, simulate exposure of fluids or slides to laboratory conditions over a long period.

The first lot of commercial hematein produced by this method, lot No. FHi-l of MacAndrews and Forbes, has just been put thru the staining test and it gave splendid slides. Our regular routine procedure is as follows:

1. Sections, paraffin or celloidin, of Bouin or Zenker-formol material, are run down to water and put in hemalum until properly stained (5 minutes usually).

2. Rinse 1 to 3 seconds in tap water. If the rinsing is quick, some alum is carried over into the eosin. This brings about a precipitate of eosin in the tissue, which is not easily removed by subsequent treatment. This, I consider, an advantage. The eosin solution must be filtered from time to time and replaced when too weak, in as much as the alum causes a precipitate to form.

3. Dipped 1 to 3 seconds in eosin bluish (one part stock to two parts distilled water). The stock is 0.5% dry powder in 20% alcohol.

4. Washed well in several changes of tap water, or in running tap water several minutes.

5. Dehydrate and mount.

For celloidin sections unfixed to slide, these are run up to 95% alcohol, spread on slide, blotted smooth, wet from a dropping bottle with absolute alcohol, drained off and mounted in euparal. This does away with creosote and greatly hastens the work.

I trust that the foregoing directions will help to save time for others, as it has for us, and give good results. The procedure is simple enough for the uninitiated and good enough for the initiated. Our congratulations are due to MacAndrews and Forbes for their splendid hematein.

THE MECHANISM OF STAINING EXPLAINED ON A CHEMICAL BASIS

II. General Presentation

Allen E. Stearn and Esther Wagner Stearn

Division of Physical Chemistry and Department of Preventive Medicine, University of Missouri, Columbia, Mo.

Abstract: **At present the division of the factors influencing staining into those supporting a chemical mechanism or those supporting what is known as "adsorption" mechanism is questioned because the latter term cannot be definitely defined from general usage. In this paper the nature of adsorption is discussed. Data indicating definitely a chemical mechanism are not directly considered; but in many cases often cited by other writers as necessitating a non-chemical mechanism, the validity of such mechanism is questioned.**

A bit of confusion, or at least lack of agreement, as to staining mechanisms seems to lie in the fact that, consciously or unconsciously, the term adsorption is ordinarily assumed to involve forces non-chemical. In the recent paper in this journal on the mechanism of staining by Holmes (1929), the title and the text lead one to this feeling, tho not explicitly so stated in any portion of the text. In the present paper, the authors propose to deal with the subject in a general way seeking to do two things: first, to inquire what adsorption signifies, and second, to consider a few arguments which have been proposed against a chemical theory of staining, since most of the arguments for any so-called physical theory consist ordinarily of objections to chemical mechanisms rather than definite suggestions of any physical mechanism.

Probably the most conservative definition of what most of us really mean by adsorption is given by Michaelis (1926) who states (p. 232 *et seq.*) that adsorption is "that process by which a substance accumulates at a boundary surface of two contiguous phases in a concentration higher than it exists in the interior of these two phases. The question of the forces causing this accumulation is deliberately left open, i. e., it is immaterial for the present whether it is caused by physical or by the so-called chemical forces. At the time when Rutherford's atomic model is constantly furnishing greater support for the common basis of the physical and chemical properties of matter, it appears quite futile to attempt to differentiate between mechanical forces of adhesion and chemical attractions. We shall therefore use the term adsorption not in any sense of distinction from

chemical union, and shall take it to include cases which theoretical chemists would recognize as chemical reactions, as well as such cases which, to an investigator of the older school, would not convey the least suggestion of chemical affinity."

Insoluble adsorbents he divides into: *acidoids*, which adsorb OH-ions or give off H-ions while maintaining a negative charge, or adsorb H-ions on neutralization of their negative charge; *basoids*, which would show behavior analogous to bases similar to that of acidoids in its analogy to acids; and *amphyloids*. "In adsorbing a dissolved base an acidoid forms a salt-like surface compound." Michaelis is well aware of the fact that the phenomenon of adsorption "will be regarded for a long time to come in the light of a process quite distinct from those of ordinary chemical reactions," but in the light of present knowledge thinks it would appear quite appropriate to do away with such limitations. When he speaks of adsorptive processes he states that "it should be clearly understood that *they do not represent a fundamental contrast to chemical union*," tho since the reaction is limited to the boundary surface of two adjacent phases, "the isolation of the resulting compound in a pure state is not possible, and therefore, its stoichiometric relations cannot as yet be established."

The type of adsorption which is of most interest in biological staining is that in which the adsorbent is itself capable of forming ions, since the proteins, nucleic acids, etc., which are typical of protoplasm, can form such ions, as is well known. The preceding paper in this series (Stearn, A. E. and E. W., 1929) presents data to show that, for certain typical protoplasmic constituents and dyes, a type of reaction, or adsorption, takes place which Michaelis would term "adsorption by exchange." A very simple case is the adsorption of eosin by ferric oxide sol, or of methylene blue by kaolin. These reactions proceed "along the lines of ordinary chemical laws, and here it would be quite futile to attempt to demonstrate any contrast between chemical reactions and adsorptions." In fact "such adsorbents behave entirely according to the well-known laws of chemical reactions and react with electrolytes in aqueous solution by means of an exchange of ions. A solution of methylene blue chloride reacts with kaolin according to the following scheme:—alkaline earth silicate + methylene blue chloride = methylene blue silicate + alkaline earth chloride." The driving force in this reaction is the low solubility of the dye compound. The formation of an un-ionized compound would furnish one just as effective. Dye-protein compounds are insoluble under certain conditions and soluble under

others, but the data in the preceding paper, as well as other work,[1] indicate that the compounds between dyes and typical protoplasmic constituents are often highly un-ionized. From the work of Rona and Michaelis (1920) on the action of dyes on cellulose, even after attempts to get the latter material as electrolyte-free as possible, it was found that basic methylene blue chloride dyes the cellulose, the positive dye ion replacing Ca and the chloride from the dye going into solution as $CaCl_2$. The NH_4 salt of eosin reacted with cellulose, dyeing it with the negative eosin ion while the NH_4 was found in solution as neutral salt. All these reactions are simple metatheses, even tho they take place at a surface between two phases. The probability of such mechanisms when stain and the stainable constituents or components of protoplasm are dispersed thru the protoplasmic substrate should seem at least as great.

Let us now consider a few objections to such a mechanism. We quote from the final foot-note of the article by Holmes (1929) in which the conclusion is reached from an extensive study by Yuri on bacterial staining that the "general character of bacterial staining is one of adsorption in which electrostatic affinity plays the principal role." The term of interest is electrostatic affinity, which, since it is taken explicitly in support of a physical mechanism thesis, must be taken to mean a non-chemical or non-specific electrostatic affinity, and one is thus almost driven back to a coulomb attraction. If we follow where this leads we are at once in difficulty, however, for such attractions depend only on magnitude of charges and the distance between them. Other things being the same, dyes in ionic form with their ionic radius large and their valence ordinarily unity, should not be retained even in the presence of simple monovalent ions of the same charge, and such staining in the presence of simple polyvalent ions should be out of the question. If the dyes are in colloidal suspension their adsorbability due to increased radius, should be even less than when ionically dispersed, and tho the fact that there might be large particles settling out and apparently coloring a surface might indicate staining, yet even gentle decolorizing would remove these. On the other hand, if one is forced to a type of electrostatic affinity which is specific, one can hardly claim to be dealing with a non-chemical force unless one shows that such specificity is correlated with ionic radius or some other physical property of stain which would permit of a physical interpretation. In this connection the question of the behavior of dye mixtures is apropos.

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[1]Soon to be published in *Journal of Bacteriology*.

The fact of differential staining with dye mixtures has been mentioned in support of a physical staining mechanism, tho if the forces were purely physical as those given above, and non-specific, it is difficult to account for such a phenomenon when the dyes are of the same class. On the other hand, such action is by no means "inconsistent with accepted conceptions of chemical reactivity." We have *chemical* analogies in large number. Suppose, for example, we have equal concentrations of two acidic ions, say acetate and cyanide, with H-ion available for only one. Both acetic and hydrocyanic acids are weak, nevertheless they will *not* divide the available H-ions equally between them. If their respective ionization constants differ by 10^4 or more, the one will be completely saturated with the H-ion (99%) before the other gets any (1%). In the same way there is no reason to assume the same ion product constants for the components of tissues with different dyes even tho they are of the same class; in fact it would be strange if one found it true. In the above chemical example the H-ion would play a role analogous to adsorbent with the negative ions analogous to two acid dyes.

It seems to the present authors that some light on general dyeing behavior might be obtained by considering the function of decolorizers. The general behavior of decolorizers has received little but technical study. In this connection the authors cannot find themselves in sympathy with terming alcohol an inert decolorizer as Holmes does (1929,p.79). The writers (1928 b) have indicated pretty clearly that the lower alcohols, as well as other acidic compounds such as phenol, cresol, etc., are much more effective in removing basic dyes, more or less regardless of solubility of the dye in them, than are basic decolorizers. Basic decolorizers such as anilin, etc., are especially active in removing acid dyes. An inert decolorizer in this sense is hard to find. This general behavior of decolorizers confirms not only the chemical nature of the act of removing dye from whatever form it is in when combined with protoplasm, but also gives indirect evidence of the chemical nature of that binding,

Perhaps it would be well to inventory the possible physical mechanisms of staining, since most writers favoring such a theory present very little that is definite, except the use of the term adsorption which we have seen is at least not a non-chemical term. There are but four obvious ones which occur. One is an electrostatic non-specific coulomb attraction. Some of the inadequacies of this mechanism were suggested above and many others will occur to one. Another is the concentration in the surface layer of any substance which decreases surface tension. Assuming the absence of specific

chemical forces, this concentration behavior has been formulated by Gibbs in his well-known adsorption formula. It is just as well known, however, that one must search long for a system which obeys this formula. The equation is thermodynamically sound but assumes the *absence* of specific chemical forces and is found experimentally inadequate. The conclusion is obvious. The third purely physical mechanism is one proposed by certain authors to explain the Gram reaction and is a sort of sieve theory in which a dye molecule enters a membrane, is increased in size by a mordant and cannot escape. A more or less extensive discussion of the peculiar consequences of this picture has been given by the authors elsewhere (1928 a), but it might be added in light of the data presented in this Journal (Stearn E. W. and A. E., 1929) on the staining of certain granules in starved bacteria that a very intricate network of such membranes would be necessary to explain some findings. Finally, a distribution mechanism in *some few cases* is one which may be considered largely physical and which may seem satisfactory under proper conditions. Even here it should be kept in mind that the distribution coefficient for efficient staining must be large or the enormous exeess of decolorizer ordinarily used will easily decolorize the tissue, if the stained tissue is permeable to it and if the dye is soluble in it. It is also hard even here to get away from chemical forces, since very often it is found that in cases of great solubility we run into definite combination with the solvent as in the cases of hydration of NH_3 and HCl. Such a mechanism, however, may be the one often effective in fat staining, since many stains are not very soluble in ether and many ordinary decolorizers do not rapidly permeate fats. In this manner the fat and wax content of tubercle bacilli probably determine their staining behavior in the Ziehl-Neelsen technic.

It might be well finally to mention one or two observations which have been used as evidence against a chemical mechanism, but which, upon inspection, seem to be of such a nature as to give no unequivocal indications one way or the other. In the article by Holmes (1929, p. 77) occur the two statements: "Fats are frequently stained, for example, by means of dyes which may be considered chemically inert. The bacteria of soils, again, are stained effectively by certain metallic salts of the dyes of the eosin group, whereas staining is ineffective with other metallic salts of the same dyes which would enter into chemical reaction with equal facility." While it appears highly possible that a distribution theory may be found to cover many fat-staining reactions, the question might be

well asked what is the basis for judging a dye, or any compound, chemically inert? Most salts in solution are mutually inert, e.g., there is no evidence of reaction between NaCl and KNO_3. But there are many exceptions. Mercuric mercury is determined quantitatively by titration with sulfocyanate, not due to insolubility of the mercuric sulfocyanate but due to the fact that it is un-ionized. Thus such a salt as NH_4CNS, tho ordinarily metathetically inert, is not so to mercuric nitrate. Similarly we dissolve $PbSO_4$ in $NH_4C_2H_3O_2$ due to one small ionization of lead acetate.

The difference in intensity of staining with different metallic salts of the same dye is of great technical importance, but unless it can be shown that this is due to a difference in amount of dye held by tissue it can have little bearing on mechanism. In the case cited it can be shown that even a solution of eosin in the presence of KCl and one of the same dye concentration in the presence of $BaCl_2$ are different in appearance, so that more definite work will be necessary before interpretation of this type of result in terms of mechanism is justified.

A bulwark of strength for proponents of a physical mechanism of staining is the well-known adsorption formula of Freundlich. However, regardless of whether this formula represents experimental results, it can be easily shown that it tells nothing of mechanism. This conclusion has been corroborated by more than one colloid chemist in conversation with the authors.

We write it in the form

$$X = KC^{1/n} \qquad (1)$$

Since X is interpreted as mass of material adsorbed per unit amount of adsorbent, it has the dimensions of concentration. Thus we can write the equation

$$\frac{X}{C^{1/n}} = K \qquad (2)$$

which is the form of the general distribution law. A *distribution mechanism* is just as good an empirical interpretation of an *adsorption* as any other.

It seems to be generally assumed that where the value of 1/N in the adsorption formula is unity the reaction is chemical, otherwise it is an "adsorption."[2] While the authors are certainly in agreement with the first part of the above conclusion, there is no more justifi-

[2]See for example A. D. Hirschfelder and H. N. Wright. Reaction between Antiseptics and Proteins, and Antiseptic Activity of the Adsorbed Portion. Proc. of Soc. for Exp. Biol. and Med. **26**, 787. 1929.

cation for it *from the formula* as representing the mass law, than there is in assuming that any value of 1/N differing from unity does not represent mass law. The simplest case, neglecting ionic exchange, of such a reaction would be represented by

$$A + B = AB$$

where A is adsorbent and B adsorbed material. Mass law would demand

$$\frac{(AB)}{(A)\,(B)} = K \text{ in place of } \frac{(AB)}{(B)} = K, \text{ from the adsorption for-}$$

mula, i.e. as adsorption takes place the available surface does not remain the same.

On the other hand, a few simple calculations will indicate that the course of a typical neutralization reaction can simulate a typical adsorption. Take a solution of the salt of an acid whose ionization constant is 10^{-3}, for example, and add strong acid, measuring the quantity of H-ion "adsorbed" or bound for any final H-ion concentration. Table 1 gives a few values calculated from the mass law for a molal solution of such a salt.

TABLE 1.—SHOWING THE RELATION BETWEEN H-ION "ADSORBED" BY A MOLAL SOLUTION OF AN ANION WHICH FORMS A SPARINGLY IONIZED ACID AND THE FINAL H-ION CONCENTRATION.

(H)	Bound H-ion grams	K in Freundlich equation	
C	X	For $\frac{1}{n}=1$	For $\frac{1}{n}=.37$
7×10^{-5}	.0654	935	2.25
10^{-4}	.091	910	2.76
3×10^{-4}	.231	770	4.66
7×10^{-4}	.412	590	6.07
10^{-3}	.50	500	6.46
3×10^{-3}	.75	250	6.41
7×10^{-3}	.876	125	5.46
10^{-2}	.909	91	5.0
3×10^{-2}	.968	32	3.55
7×10^{-2}	.986	14	2.64
10^{-1}	.99	10	2.32

Here it is seen that K varies from 10 to 935 for 1/n = unity but only from 2.25 to 6.46 for 1/n = .37. It is true the equation does not strictly represent the formula, but H. N. Holmes (1922) makes the general statement that tho "the logarithm of the equation should plot as a straight line, it usually misses this ideal somewhat." He

further states that "adsorption is proportionally greater for very dilute solutions than for concentrated ones, which is certainly not true of reactions." And yet here is a reaction of such a nature that it should *not* be a reaction according to the above criterion and yet is one of the most unequivocal of examples. For dilute solutions from (H) = 10^{-4} to (H) = 10^{-3} the "adsorbed" hydrogen ion increases by 5.5-fold, while from (H) = 10^{-2} to (H) = 10^{-1} the increase is less than 1.1-fold. It should be noted that the concentration range of H-ion, $7x10^{-5}$ to 10^{-1}, is greater than ordinarily studied. If X be plotted against C, a curve looking like an adsorption curve is obtained. Naturally a choice of coordinates will make an "adsorption" curve approach the conventional "reaction" curve and vice versa, which again leads to the conclusion that "adsorption" behavior throws no light on mechanism.

The authors have no quarrel with the word adsorption. It is very convenient, but should not be taken to signify a union involving essentially non-chemical forces, nor should one get the feeling that its use constitutes an explanation of mechanism.

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THE NEGATIVE STAINING OF BACTERIA

W. Ch. Dorner,

Swiss Experiment Station for Dairying and Bacteriology, Liebefeld-Berne, Switzerland[1]

Abstract.—**The author does not propose new methods but calls attention to the value of Burri's India ink technic and Fischer's use of nigrosin for the negative staining of bacteria. The latter is particularly useful. The technic is as follows: Boil 10 g. nigrosin in 100 cc. water about 30 minutes. Filter several times thru the same filter paper, adding 10 drops of formalin before the last filtration as a preservative. Place a small loopful of this solution on a clean slide and add the bacteria with a needle or loop. After mixing spread the mixture a little irregularly on the slide and dry either at room temperature or slowly over a low flame. The preparation may be examined in oil immersion without the use of a cover slip.**

The purpose of this paper is not to introduce new methods but to bring about greater familiarity with an old type of technic for staining bacteria which is often very useful.

In his first publication on his India ink technic Burri[2] coined the expression "negative staining" for a procedure involving the application of a liquid that dries on the slide forming a uniform dark film but not staining the bacteria. The latter are then visible as white corpuscles in a dark field. In principle, the negative staining of bacteria consists in the production on the slide of an opaque film which in an ideal case has exactly the thickness of half of the diameter of the bacteria. It does no harm if the film is thinner, as long as the bacteria are visible, because of the capillarity which will attract enough dye around the bacteria to let them appear the right size. If, however, the film is too thick the bacteria will seem smaller or even be covered completely, consequently remaining invisible.

Altho described earlier, the use of India ink for staining bacteria has become more common since the above mentioned work of Burri. Later Fischer[3] proposed to use nigrosin B, Grübler. For the purpose of negative staining of bacteria nigrosin is better than India ink because, being a solution, it gives a perfectly homogenous background, while there are always fine granules in the India ink.

[1]Temporarily at the New York State Agricultural Experiment Station, Geneva N. Y. as fellow of the International Education Board.

[2]Burri, R. Das Tuscheverfahzen, Jena, 1909.

[3]Fischer, H. Zts. Wis. Mikr. **27**, 475.

Theoretically the negative staining is applicable to all bacteria. It must be admitted, however, that with fluids containing much organic matter like pus, or to some extent milk, the results are often rather unsatisfactory. The casein and the leucocytes are stained by the nigrosin which sometimes covers the bacteria. In whole milk most of the trouble comes from the fat globules which do not take the color, but being of greater size make many bacteria invisible. India ink does not color the leucocytes and the casein in this way, but it has the same inconvenience as nigrosin in relation to fat globules.

The nigrosin solution is prepared as follows: 10 g. nigrosin are boiled in 100 cc. water for about 30 minutes in order to dissolve the dye as completely as possible. The solution is then filtered several times thru the same filter paper. The purpose of the filtration is to remove any solid particles which may be contained in the dye. Before the last filtration it is well to add 10 drops of formalin in order to prevent any further bacterial growth in the solution.

A good nigrosin solution should not contain any visible dirt particles or bacteria. The film obtained by spreading some of the solution and drying it should appear perfectly homogeneous when examined with an oil immersion objective. The nigrosin provided by Grübler in Leipzig fulfils these requirements. At first poor results were obtained with the nigrosins made by Coleman and Bell and by the National Aniline & Chemical Company because of their dirt content, but later samples by these same companies (certified by the Stain Commission) were found to be equal in quality to the Grübler product. If the procedure is to be employed to a great extent in staining bacteria picked from colonies or agar slants, it is advisable to dilute the above nigrosin solution with an equal quantity of water. For making preparations from liquid cultures, however, it is preferable to use the 10% solution, because in this case the nigrosin is diluted by the liquid suspending the bacteria.

Nigrosin cannot be used for very acid cultures. An acidity to phenolphthalein of 1% in lactic acid in ordinary culture media is probably near the limit within which preparations can be made successfully. At a higher acidity the nigrosin precipitates. With such cultures, it is necessary to neutralize the medium before staining. India ink is much more sensitive to acidity than nigrosin.

The making of a preparation with India ink or nigrosin is very easy. One small loopful of nigrosin is put on a clean slide, then the bacteria are added with a needle or loop. After mixing gently the liquid is spread not too regularly by means of the loop or the needle,

and then dried. In spreading a little irregularly there are places where the film will be thick and places where the film will be thin. It is then easy to choose the right spot for examination. The drying can take place at room temperature or slowly over a pilot flame. Too rapid drying and too concentrated solution of nigrosin sometimes causes the formation of cracks in the preparation. The cedar oil can be put directly on and the preparation is ready for examination. The oil can be removed with xylol without damaging the preparation.

The nigrosin technic has great advantages in comparison with the staining methods ordinarily used. The rapidity with which a preparation is made is one factor. No staining technic is as rapid as the negative staining. It should, therefore, be always employed when the staining is only to make bacteria more visible.

There is no fixation of the bacteria previous to the staining. Therefore the chances are greater of seeing the bacteria in the preparation in their natural state. There is no doubt that the usual fixing in a flame as a roasting process generally causes changes in their size and possibly in their form. As the negative staining avoids fixation, the pictures obtained are nearer to reality.

Another advantage of the nigrosin technic is that many bacteria of the Gram-negative group have a characteristic appearance when thus stained. Many Gram-negative rods show a little dark dot in their center. If this dot is visible one can be sure that the organism is Gram-negative (altho these spots are not always present in an organism of this group) for it has never been observed in the Gram-positive organisms.

The simplicity of the nigrosin stain is another advantage. There is no possibility of overstaining bacteria. Beside the difficulties encountered in the application of the negative staining for acid solutions and media with too much organic matter, it must be admitted that this method is not very satisfactory for staining larger organisms like yeasts, etc. The reason for this is that the liquid collects around the cells thru capillarity forming dark places there.

NOTES ON TECHNIC

DETECTING OIL IN TWIGS.—During the process of investigating the penetration of oil into dormant twigs it was found necessary to develop a technic for demonstrating the oil after it had penetrated. The technic used is as follows:

1. Cut sections of the twigs on the hand microtome about 20μ thick cutting the sections dry in order that the oil will not be displaced by water any more than can be helped.

2. The sections are transferred directly to a saturated solution of sudan IV[1] in equal parts of 70% alcohol and acetone, and are stained for one minute.

3. Wash briefly in water.

4. Transfer to a solution of methylene blue[1] (Unna's polychrome methylene blue, 1 pt.; water, 9 pts.) and stain one minute or until the tissue is faintly blue.

5. Wash in water, place the sections on a slide, remove the excess water with a blotter or filter paper and mount in glycerin.

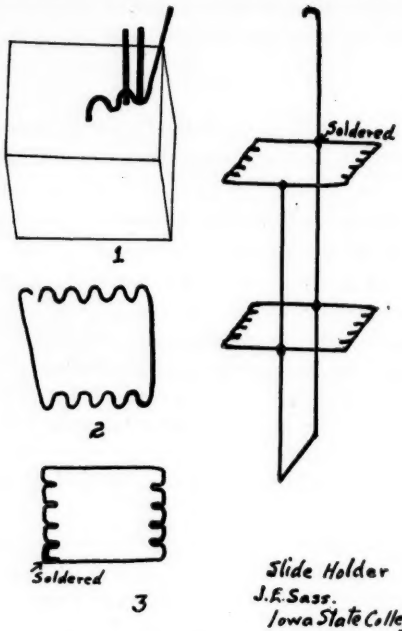
By this method the spray oil is stained a brilliant red and may be distinguished as large droplets with the 16 mm. objective and 10 × eye piece. If the sections are examined with the oil immersion lens it is observed that in many cells of the tissue there are many minute red staining globules, and these are also present in the control tissue and are small droplets of natural fat. They stain in exactly the same manner as the spray oil, and for this reason tiny droplets of spray oil, if there are any present, cannot be distinguished from the natural oils by this method.

However, if the low power is used in examining the slides as is mentioned above, the tiny droplets of natural oil appear only as a pink haze, whereas the large droplets of the spray oil stand out strikingly against the rest of the tissue. Such large droplets were never seen in the control, unoiled twigs.—RACHEL HAYNES, Geneva, N. Y.

A WIRE HOLDER FOR STAINING SLIDES.—There are several types of slide-staining holders on the market. Most of them are of sheet metal and are open to the objection that they carry over too much reagent, because of the large metal surface exposed. We have been using a wire holder which minimizes this fault. Our holder is much

[1]The dyes employed were stains certified by the Commission. The methylene blue had a dye content of about 90%.

easier to make than its appearance may suggest. The procedure is as follows: Drive two one-inch brads into a hardwood block, just far enough apart to allow 20 gauge copper wire to slide between them. By bending the wire over the nails as shown in Fig. 1, make a series of ten loops. Skip a space equal to the width of a slide and make another series of loops, Fig. 2. Close the outside loops and square up the inside loops with a pair of thin-nosed pliers, and solder as indicated, Fig. 3. Two of these pieces are soldered to an 18-gauge copper frame which is extended to form a handle, Fig. 4. The holder illustrated carries five slides and fits a Coplin staining jar. A larger holder can be made to hold ten slides and fit a screw-top capsule jar. This holder cannot be used with iron-hematoxylin because of excessive galvanic action at the soldered joints, but it is satisfactory with hemalum and the anilins. Perhaps a holder made of monel metal with spot-welded joints would be usable with other stains and reagents. This holder saves time in the routine preparation of slides for general class use and in certain operations in research work.—JOHN E. SASS, Ames, Ia.



MEYER'S HEMALUM—A CORRECTION.—Thru an oversight in the manuscript the formula published on the first page of the writer's paper on Meyer's hemalum (Stain, Techn. **4**, 127–9. 1929) was incorrect. It should call for 0.2 g. $NaIO_3$ instead of 1 g. The correct formula is:

Dissolve 50 g. alum in 1 litre of boiling water. Add 1 g. hematoxylin, and 0.2 g. $NaIO_3$. Cool and filter.—J. E. SASS, Ames, Ia.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

CHRISTELLER, E. and KAISER, K. A microchemical histological method for differentiation of tissues by means of ferric salts formation. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 115–117, 1929.

Ferric salts proved successful for fixing acid ingredients of tissues, in this case also lactic acid (Otto Warburg). Operation material (carcinoma and normal tissues of different internal organs, skin, and from amputated limbs), and guinea pigs were used.

Method: Immerse small slices of *fresh* tissue in solutions of the following iron salts—ferric chloride, ferrous chloride, ferric lactate, ferric sulfate, iron iodide or ferric ammonium sulfate for 24 hours. Make frozen sections right away, or add formalin and leave for another 24 hours. Wash in distilled water, treat with potassium ferrocyanide plus HCl. The ferric-ferrous salt formation takes place in all cases.

Results: 1. Connective tissue takes hardly any iron and appears pale;
2. Nuclei of all cells show a weak blue;
3. Red blood cells are pale green, plasma in the vessels light greenish;
4. Epithelia show a dark blue protoplasm;
5. Carcinoma cells show the highest degree of impregnation;
6. Smooth and striated muscle are light blue.

Counterstain with eosin, van Gieson, tartazin, orange G, etc. Especially good with carmin and phenosafranin.

Ferric salts which precipitate proteins of the blood serum *in vitro* give the most intensive picture. Ingredients of the protoplasm which react with iron salt solutions are probably mostly globulins and albumins. Impregnation is probably partly due to a precipitate of iron albuminate. Another greater part of the precipitate is of some other nature because ferrous salts also give distinct pictures.

LILLIE, R. D. A brief method for the demonstration of mucin. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 120–121, 1929.

The method has been tested on normal and pathological tissues fixed in formalin or Zenker-Helly.

1. Transfer paraffin sections thru xylol and alcohols to water. Treat with iodine and with sodium thiosulfate if the fixative used contained mercury.
2. Transfer sections to a 2% aqu. sol. of toluidin blue (no information given as to source or dye content) for 1 minute.
3. Wash in water, dehydrate in pure acetone (alcohol does not preserve metachromasy as well), clear in xylol and mount in neutral balsam.

Results: Mucin—reddish violet; cell nuclei and bacteria—deep blue; red cells—yellow or greenish yellow; cytoplasm, fibrous tissue—bluish green; thyroid colloid—very pale blue; decalcified bone—light bluish green with pale violet Sarpey's fibers; cartilage matrix—deep bluish violet; hyaline and amyloid—bluish green; caseous matter—pale blue green; coagulated serum—pale greenish blue; muscle light blue; and most cell granules are blue violet.

SHERIDAN, W. F. Weigert's elastic tissue stain prepared with crystal violet. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 123, 1929.

The advantage of crystal violet over basic fuchsin in the preparation of Weigert's stain, is its definite chemical composition, greater stability, complete selectivity and better differentiation in the presence of blue counterstains. Elastic fibers stain green to greenish black.

Method: Crystal violet (source or dye content not specified) 1.0 g.; resorcin, 2.0 g.; distilled water 100 cc. Dissolve by boiling a few minutes, stirring constantly in a porcelain capsule of about 300 cc. capacity. Add to the boiling mixture 30 cc. of a 30% aqu. sol. of ferric chloride. Continue boiling, stirring constantly, for several minutes or until precipitation ceases. Collect precipitate on filter and wash with 50 cc. of distilled water. Dissolve in 100 cc. of absolute alcohol by boiling in porcelain capsule on electric stove or sand bath. Cool, filter and add 2.0 cc. HCl. Bring paraffin sections to alcohol and stain one or more hours. Differentiate in absolute alcohol until elastic fibers alone remain stained. After several hours of staining there is no appreciable loss from prolonged differentiation.

BRILMYER, G. J. Combining a nuclear and differential tissue stain. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 122, 1929.

This is a combination of Delafield's hematoxylin and Mallory's connective tissue stain. The hematoxylin intensifies the nuclei and is in turn converted over to a red color by the action of the acid in the connective tissue stain. The method does not require specific fixation.

1. Stain in Delafield's hematoxylin for 5 minutes, wash.
2. Stain in 0.2% aqu. sol. of acid fuchsin for 1 minute, wash.
3. Stain for 2 to 3 hours in:

Anilin blue (water sol)	0.5 g.
Orange G	2.0 g.
Phosphomolybdic acid, 1% aqu. sol	100.0 cc.

(Source or dye content of stains not specified)

4. Wash and pass rapidly thru 35%, 70% and 95% alcohols. Dehydrate in absolute alcohol. Clear in xylol and mount.

INGLEBY, H. A modification of Achúcarro's method of staining neuroglia. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 91–95, 1929.

Demonstration of fine fibrils of the podics of fibrous neuroglia, (Cajal's fibrous astrocytes.)

Method:

1. Fixation.
   a. Cajal's formol bromide: Ammonium bromide—2.0 g., formalin—14 cc., distilled water—84 cc.

2. Use thin slices of fresh tissue (best 6 hours within death), with evenly cut surfaces. Fix 2 to 4 days for astrocytes in the white matter of the hemispheres, 18 hours to 2 days for marginal glia, 7 to 10 days for spinal cord.
   b. Picric acid, sat. aqu.—75 cc., acetic acid—5 cc., formalin—25 cc., ammonium bromide—2 g. Make up fresh. Dissolve ammonium brom. in formalin, add picric and acetic. Fix 2 to 24 hrs. Best for marginal and protoplasmic glia.

2. Rinse, freeze and cut sections 15 μ thick. Transfer to water, add 2 to 3 drops of ammonia.

3. Wash and pass one by one to 10% tannic acid. Warm 5 to 7 minutes at about 50° C. Cool and leave in the acid 10 minutes to 2 hours.

4. Transfer to water, add ammonia (3 to 6 drops to 10 cc.). Move sections when they recover their pliability, wash.

5. Transfer to silver bath (Da Fano's modification of Bielchowsky's method). Add 2 drops of 40% sodium hydroxide to 5 cc. of 20% silver nitrate. Shake well, add ammonia until precipitate is just redissolved, add extra drop. This keeps 3 to 4 days in dark. Add 2 cc. of this to 30 cc. of water in a flat glass dish. Spread sections and move them occasionally. Change solution if yellow discoloration is excessive. Sections get yellowish brown.

6. Transfer to 20% formalin; sections get brown, greenish brown in few minutes; wash twice.

7. Tone in gold chloride, 1 in 500, fix in 5% hyposulfate of soda, wash, transfer to 50% alcohol.

8. Mount, blot, dehydrate with absolute alcohol; clove oil or origanum oil; xylol; Canada balsam.

If overstained decolorize with potassium cyanide (2% aqu. sol.). Destaining however should be avoided as finer details are liable to get lost. Perivascular connective tissue also stains.

HAYTHORN, A. R. The decolourization of tubercle bacilli in sections at low temperatures. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 130–134. 1929.

The solutions used in fixing, embedding, dehydrating and mounting paraffin sections remove something from tubercle bacilli that is necessary for maintaining their acid-fast qualities. Zenker's fluid is better in this respect than formalin; formalin fixation should be used only when frozen sections are to be stained within 48 to 72 hours. For such frozen sections and for material fixed in Zenker's fluid (containing 5% acetic acid), using 5 to 10 times as much fluid as there is tissue to be fixed (followed by the usual steps of dehydrating, embedding and sectioning), proceed as follows:

Stain lightly (2 to 5 minutes) in hematoxylin; decolorize in acid alcohol if overstained; place in tap water until blue. Stain in Ziehl's carbol-fuchsin one hour in paraffin oven at 55°C; wash in tepid water, then in ice water (8 to 10°C) decolorize in 10% sulfuric acid, cooled by standing several minutes in ice water, until sections are pale violet; wash in ice water and repeat application of acid if too much red color returns; wash in ice water and stand in tap water until blue; remove sections one at a time, blot, wash rapidly with 95% alcohol from a dropping bottle. Flood with orange G dissolved in absolute alcohol, from a drop bottle, until section is pale orange; wash with absolute alcohol; blot and flood with xylol; blot and mount in balsam. (Writer does not specify source or dye-contents of stains used.)

CAMPBELL, HELENOR. A stain for *B. leprae* and myelin sheath. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 129–130. 1929.

To stain the neurokeratin of myelin sheaths at the same time as demonstrating leprosy bacilli proceed as follows:

Fix tissue in acetic Zenker. Wash. Place for 6 to 24 hours in equal parts of 80% alcohol and Lugol's solution. Place in 80% alcohol 12 to 24 hours; 95% alcohol 2 to 6 hours; absolute alcohol two changes, 6 to 24 hours; absolute alcohol and xylol (equal parts), ½ hour; xylol ½ hour; paraffin two changes 1 hour. After sectioning and securing to slide, place in xylol to remove paraffin; stain ½ hour in Kinyoun's carbol fuchsin (basic fuchsin 4 g., phenol crystals 8 g., 95% alcohol 20 cc., distilled water 100 cc.) Rinse in water; acid alcohol (0.5% HCl in 35% alcohol), two changes; do not completely differentiate at this step. Stain in Harris' hematoxylin without acetic acid 2 minutes or less. Differentiate again in the acid alcohol; rinse in water; place in 1% ammonia water; rinse in water. Counterstain in 1% aqu. sol. orange G. Dehydrate quickly in two changes of acetone; xylol; mount in xylol-damar or xylol-balsam. (No information given as to source or dye-content of stains used.)

The leprosy organism and the neurokeratin stain red, nuclei blue and cytoplasm yellow.

HAYTHORN, S. R. A hematoxylin erythrosin Gram-Weigert stain. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 128–9. 1929.

A method is given for combining hematoxylin and erythrosin with the Gram-Weigert technic, for demonstrating fibrin and the Gram-positive bacteria. It is applicable to both formalin and Zenker fixed material, is simple to use and gives permanent preparations. The technic is as follows:

Sections are brought to water in the usual way. Stain in Mallory's alum hematoxylin 5 to 10 minutes, or longer if Zenker material is used. Rinse quickly in acid alcohol. Wash. Allow to stand in tap water 2 to 5 minutes. Stain in Weigert's anilin methyl violet 2 to 5 minutes. Blot. Cover with Lugol's iodine solution 2 to 5 minutes. Blot dry. Decolorize in two parts anilin oil with one part xylol. Flood with erythrosin (sat. sol. in absolute alcohol), for 30 seconds to 1 minute. Wash off with one part anilin oil in two parts xylol. Wash with xylol; blot and mount. (No further information as to source or nature of dyes is given).

The nuclei are blue; fibrin and Gram-positive bacteria are purplish blue; while the diffuse stain is reddish pink.

CHASE, W. H. A rapid frozen section method for fresh tissues. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 126–127. 1929.

The method is applicable for ordinary and special stains. Sections are fixed on the slide by a 0.2% celloidin solution which completely permits dehydration and clearing without causing irregularities and artefacts inevitable when sections are mounted after dehydration. The technic is simple, reliable and permanent.

1. Cut sections of fresh or formalin fixed tissue as thin and even as possible in the freezing microtome.
2. Float sections on slide and dry slowly with aid of a little heat.
3. Dip the fixed slide into a 0.2% celloidin solution and plunge into a dish of cold water.
4. Stain in hematoxylin, 30 seconds to 1 minute.
5. Wash in water. Place in lithium carbonate for 30 seconds, wash in water 30 seconds to remove all lithium carbonate.
6. Stain in eosin (source or dye content not specified), for 1 to 2 minutes. Wash in water.
7. Dehydrate in 95% and absolute alcohols.
8. Dip in ether to remove the celloidin film, and put back into absolute alcohol for 10 seconds.
9. Clear in equal parts of absolute alcohol and xylol, then in xylol, mount in Canada balsam.

AMBROGI, L. P. A rapid paraffin embedding method. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 124–125. 1929.

The method is especially recommended for tissues which get brittle from chloroform treatment. It ensures good penetration for various material except that containing much air (lung), and is adaptable to all usual stains.

1. Fix thin slices of tissue (2 to 3 mm.) in 10% formalin for 6 hours;
2. Wash in running water for 2 hours;
3. Acetone thru 3 changes of ½ hour each;
4. Cedarwood oil from 3 hours or until translucent;
5. Paraffin thru 3 changes of 1 hour each.

SHERIDAN, W. F. Use of (A) normal propyl alcohol and (B) low melting point paraffin in infiltration to prevent distortion and hardening of tissues. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 125–126, 1929.

Normal propyl alcohol is a good dehydrating and clearing agent. Place fixed tissue in propyl alcohol over night and infiltrate in paraffin the following day. No hardening or shrinkage takes place. There is a remarkable development and preservation of tissue colors. Fibroid uteri, skin, connective tissue tumors, and others undergoing sclerosis, also scirrhus carcinomata are easier to section when treated with this reagent.

Soft instead of hard paraffin is proposed to prevent hardening of the above mentioned tissues. Use soft paraffin, having a melting point at 40 to 43°C., for infiltration. After completing infiltration, transfer material to hard paraffin (m.p. 52 to 54°C) for 1 to 2 minutes and embed.

KISSER, J. Die Verwendung der Gefriermethode bei Pflanzlichen Objecten. Zts. Wis. Mikr. **45**, 434–441. 1928.

The freezing apparatus may be used in connection with a sliding microtome instead of the usual type of freezing microtome. Cutting at right angles is best, yet hard or uneven material requires an oblique razor. The material cannot be sectioned if insufficiently frozen, and breaks if the freezing is overdone. Cool the razor if sections freeze to it. While cutting, the razor and object are flooded with alcohol for hardening. For soft and succulent tissues the method is ideal. Deterioration of cell structures increases with higher water content. Chloroplasts suffer less than other parts. In the case of tissues that are not homogeneous, a pump may be used to replace the air with water. By this method *Elodea* leaves can be cut 15 to 20μ thick. The method is valuable for sections of leaves parallel to the surface. Pith sections one-half to one micron thick (boiled in water) are placed on the freezing box and used as foundation for the leaves. Impregnation with gelatin or gum arabic seems better than the air pump treatment. Kill material in alcohol or formol, wash, transfer to a weak solution of gelatin or gum arabic, place in thermostat for gradual penetration and concentration, cool, freeze onto pith foundation and section. Excellent 16 μ sections were thus obtained from leaves of *Peperomia* and especially of *Dracaena Draco*.

PETRAGNANI, G. Metodi rapidi di colorazione dei corpi del Negri. Boll. Istit. Sierot. Milanese, **7**, 557–561. 1928.

The methods given are very useful for the rapid demonstration of Negri bodies, and the mordant for staining bacteria. The author's mordant is as follows: Solution I: ground potassium alum (cryst), 3 g., lead acetate (cryst) 0.5 g., glacial acetic acid 3

drops, distilled water 100 g.; dissolve on water bath and mix with: Solution II, tannic acid 7 g., ferric chloride 2 g., methyl alcohol (pure) 35 g., distilled water 15 cc. Filter after 2 to 3 days, dilute with 20 to 50 or more volumes of methyl alcohol.

Method *A* (for sections)

Run thru xylol and absolute alcohol. Treat 5 to 10 seconds in dilute mordant; wash rapidly with absolute and 95% ethyl alcohol; stain 10 to 20 seconds with good eosin ("spiritus löslich Grübler" specified, 0.5 g. in 100 cc. 50% ethyl alcohol); wash slowly with water; stain one minute with Mayer's hematoxylin (hematoxylin 1 g., sodium iodate 0.2 g., alum 50 g., water 1000 cc.); wash rapidly with water; stain with methylene blue (methylene blue Grübler 1 g., water 1000 cc.) until violet (if blue, it is overstained); dry with filter paper; wash and shake 15 to 20 seconds with absolute alcohol containing 0.25% N/2 NaOH; wash with 90 to 95% ethyl alcohol until a general blue; two changes of absolute alcohol; xylol; mount in neutral balsam. Stains nerve cells blue, nucleolus dark blue, capillaries with nucleus and endothelium brilliant red, red globules red, Negri bodies eosin red.

Method *B*

After xylol and absolute alcohol treat a few seconds with mordant diluted in 100 to 200 volumes; wash with absolute alcohol, then 75% alcohol; stain 30 seconds with eosin as above or acid fuchsin (0.5 g. acid fuchsin Grübler or Kahlbaum in 100 cc. 50% alcohol); wash in water, then in 95% alcohol; two changes of absolute alcohol; xylol; balsam. Stains cellular bodies pink; Negri bodies brilliant red. A very useful method for a rapid diagnostic stain.

Method *C*

Treat with mordant (1 vol. in 20 to 40 vols. methyl alcohol) 5 to 10 seconds; with acid fuchsin as in Method B, for one minute; wash in water; stain with indigo carmin (indigo carmin Kahlbaum 1 g., water 500 cc.) for 5 to 20 seconds; wash with water, then 95% alcohol; two changes of absolute alcohol; xylol; balsam. By this method the indigo carmin partially displaces the acid fuchsin; a good stain for demonstrating the chlamydozoic structure of Negri bodies.—*R. Ciferri.*

Moore, R. A. Gelatin carmine injections. J. Tech. Meth. and Bull. Inter. Assn. Med. Museums, **12**, 55–58, 1929.

Gelatin carmin prepared by the following method proved successful in studies of vascular changes in nephritis:

1. Let 80 g. of gelatin imbibe 200 cc. of water and then heat to complete gel.
2. Suspend 20 g. of carmin in 100 cc. of water and add ammonia until carmin dissolves.
3. Mix 1 and 2, add 15 g. of potassium iodide.
4. Place the mixture in a water bath at 25°C. and immerse the platinum electrode. Electrolytic hydrogen (from a tank) is passed over the electrode, and a motor stirrer used to agitate the gelatin. Read the electrical potential on the potentiometer by balancing against a standard cell. Cautiously add acetic acid until the reading of the voltage corresponds to a pH of 7.2.

The method is faster than Mallory and Wright's with the preparation of gelatin gel and of alkaline carmin, and the gelatin seems more uniform than when prepared by Bensley's and MacCallum's methods.

The writer states that some lots of carmin contain considerable insoluble material which must be removed; but does not state from where the satisfactory stain was obtained.

Weiss, Emil. A simple method for staining spirochetes. J. Lab. & Clin. Med. **14**, 1191–1193. 1929.

A new method for staining spirochetes has been devised as follows: The material supposedly containing spirochetes is placed on a slide in a drop of 5% glacial acetic acid. The slide is inverted over a hollow ground slide and placed in an incubator for 15 minutes. The drop is then spread and allowed to dry in the air. The slide is covered with the mordant (100 g. tannic acid dissolved in 100 cc. 95% alcohol and mixed before use with two parts of 7.5% glacial acetic acid in undiluted formalin) and steamed for 2 to 5 minutes. The slide is then washed with warm water and covered with a saturated solution of a basic dye for 2 to 5 minutes, is then washed and dried in

the air. If counterstaining is desired, cover with a 10% solution of contrasting acid dye in 30% alcohol for 8 to 10 minutes, wash with water and dry. The following combinations of contrasting basic and acid dyes are recommended: crystal or gentian violet with acid green, safranin or fuchsin with acid green, and brilliant green with acid violet or acid fuchsin. The source of these dyes is not mentioned, nor is the exact identity of acid green and acid violet specified; by the former is probably meant light green SF yellowish.

GOUGH, J. and FULTON, J. D. A new fixative for mitochondria. J. Path. & Bact. 32, 765–769, 1929.

Mercuric acetate has a definite fixative action on mitochondria. The intense staining with acid fuchsin is accounted for by the capacity of mercuric acetate to form an insoluble purple compound. The method may be used on tissue which has been in formalin even for many months. Penetration is uniform. It has been applied to kidney, liver, pancreas, suprarenal, thyroid, intestine, and stomach of rabbits and guinea pigs. In the pancreas zymogen granules are well fixed in addition to mitrochondria in the acinus cells, but no mitrochondria show in the islet cells.

1. Fix fresh, thin slices in a neutral formol-saline solution for at least 48 hours. Neutralize by adding sufficient magnesium carbonate to render the mixture alkaline. filter, titrate with untreated 10% formol saline to pH-7.
2. Wash in running water for 3 hours.
3. Mordant 48 hours in: mercuric acetate, 3 g., acetic acid 0.1 cc., distilled water 100 cc.
4. Wash 12 to 18 hours in running water.
5. Treat with 50% alcohol for 3 hours, methyl alcohol over night, absolute alcohol 3 to 4 hours, equal parts of absolute alcohol and xylol 1 to 2 hours, xylol 1 to 2 hours, paraffin wax 3 to 4 hours.
6. Stain by Bensley-Cowdry's method using 6% acid fuchsin, or 0.5% methyl green (source or dye content not specified). If overstained with the green, differentiate in 50% alcohol. Heidenhain's hematoxylin was also used.

Lugol's iodine followed by potassium thiosulfate will remove excessive mercuric acetate if necessary before staining with hematoxylin.

OKADA, S. Über eine neue Silberimpregnationsmethode zur Darstellung der Neurofibrillen. Folia Anat. Japonica, 7, 403–407, 1929.

Method for the demonstration of neurofibrils:

1. Fix thin slices of fresh tissue in the following:
   Absolute alcohol.....80 cc., 0.1% NaOH....20 cc.; or
   Absolute alcohol.....98 cc., 1% NaOH....2 cc.
   Wash in distilled water for 5 to 10 minutes.
2. Transfer to 1.5% silver nitrate sol. for 3 to 5 days at 37° C. or at room temperature. Rinse rapidly in distilled water.
3. Reduce in the following at room temperature:
   Formalin.....5 cc.; dist. water.....100 cc.; pyrogallolic acid...2 g.
4. Treat as usual, embed in paraffin, cut.
5. Transfer sections to 5% fixing natron for 5 min., and rinse in running water for few hours.
6. Dehydrate, clear and mount.

The neurofibrils should appear black on a light field, also the pericellular net of the ganglion cells. The glia fibers are not stained. Thus fixed material keeps very well.